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EXPRESSION OF GENES RELATED TO GLYCOLYTIC METABOLISM AND THEIR INFLUENCE ON MUSCLE pH IN DIFFERENT SWINE GENETIC GROUPS

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Dissertation submitted to the Universidade Federal de Viçosa, as part of the requirements of the Postgraduate Program in Animal Science to obtain the degree of *Master of Science.*

Advisor: Simone Eliza Facioni Guimarães

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RESUMO

MÁXIMO, Natanieli, S., M.Sc., Universidade Federal de Viçosa, julho de 2024. **Expressão de genes relacionados ao metabolismo glicolítico e sua influência no pH muscular em diferentes grupos genéticos suínos.** Orientadora: Simone Eliza Simone Facioni Guimarães. Coorientadores: Renata Veroneze, Giarlã Cunha da Silva

Tendo em vista que o pH muscular é um dos fatores cruciais para a qualidade da carne suína, sendo influenciado por características como a taxa e extensão do seu decaimento, raças suínas e enzimas glicolíticas. A fim de compreender os mecanismos relacionados ao metabolismo energético e à conversão do músculo em carne, o objetivo deste estudo foi investigar a existência de expressões gênicas distintas que possam explicar o metabolismo glicolítico através da análise do pH muscular, e caracterizá-la nas diferentes raças suínas. Para isso, utilizou-se 39 animais oriundos de quatro grupos genéticos: Piau (PP), Large White (LW) e dos cruzamentos Piau-Large White (PL) e Duroc-Large White (DL). Medidas de pH muscular foram coletadas em onze tempos distintos após o abate (0, 0.75, 1, 2, 3, 4, 5, 6, 7, 8 e 24 horas), o quais foram utilizados no modelo de medidas repetidas no tempo, incluindo como covariável o tempo, grupo genético e a interação tempo-grupo genético como efeito fixo, seguido de análise de médias, onde observamos algumas diferenças significativa entre os grupos (p<0.05). Enquanto que a análise de decaimento do pH foi realizada utilizando o modelo exponencial. Para análise de expressão gênica, foram utilizadas amostras de *Longissimus dorsi* de 24 animais, selecionados de acordo com a concentração de RNA total da amostra. Dez genes relacionados ao metabolismo glicolítico *post-mortem* foram selecionados, houve diferença significativa entre os grupos genéticos (p<0.05), além de observa-se um padrão de expressão, onde PP e PL, apresentaram maior expressão relativa nos genes que contribuem com a melhor qualidade da carne suína. Ademais, os animais locais demonstraram comportamento genético predominantemente aditivo, retendo algumas características favoráveis, como melhores valores de pH e uma taxa de declínio de pH mais lenta. A partir desses resultados, conclui-se que a alteração do metabolismo glicolítico através da seleção genética, ou seja, através da utilização das raças locais, tem o potencial de modular os índices de pH e, consequentemente, pode otimizar os índices de qualidade final da carne suína.

Palavras-Chave: Estresse metabólico; Músculo esquelético; mRNA; Qualidade da carne.

ABSTRACT

MÁXIMO, Natanieli, S., M.Sc., Universidade Federal de Viçosa, July de 2024. **Expression of Genes Related to Glycolytic Metabolism and their Influence on Muscle pH in Different Swine Genetic Groups.** Adviser: Simone Eliza Simone Facioni Guimarães. Co-advisers: Renata Veroneze, Giarlã Cunha da Silva

Considering that muscle pH is one of the crucial factors for pork quality, influenced by characteristics such as the rate and extent of its decline, pig breeds, and glycolytic enzymes. To understand the mechanisms related to energy metabolism and muscle-to-meat conversion, this study aimed to investigate the existence of distinct gene expressions that may explain glycolytic metabolism through muscle pH analysis and characterize it in different pig breeds. For this purpose, 39 animals from four genetic groups were used: Piau (PP), Large White (LW), and the crossbreeds Piau-Large White (PL) and Duroc-Large White (DL). Muscle pH measurements were collected at eleven different times post-slaughter (0, 0.75, 1, 2, 3, 4, 5, 6, 7, 8, and 24 hours), which were used in the repeated measures model over time, including time, genetic group, and the time-genetic group interaction as a fixed effect, followed by mean analysis, where some significant differences between the groups were observed (p<0.05). The pH decline analysis was conducted using the exponential model. For gene expression analysis, *Longissimus dorsi* samples from 24 animals were utilized, selected based on the total RNA concentration of the sample. Ten genes related to post-mortem glycolytic metabolism were selected, with significant differences observed between the genetic groups (p<0.05). Additionally, a pattern of expression was observed, where PP and PL showed higher relative expression in genes contributing to better pork quality. Furthermore, the local animals demonstrated predominantly additive genetic behavior, retaining some favorable characteristics, how to best pH values and a slower pH decline rate. From these results, it is concluded that altering glycolytic metabolism through genetic selection, specifically through the use of local breeds, has the potential to modulate pH levels and, consequently, optimize final pork quality indices.

Keywords: Meat quality; Metabolic stress; mRNA; Skeletal muscle.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABPA: Brazilian Association of Animal Protein

ADP: Adenosine Diphosphate

AMP: Adenosine Monophosphate

ATP: Adenosine Triphosphate

cDNA: Complementary DNA

DL: Duroc-Large White

DNA: Deoxyribonucleic Acid

Glu-6-P: Glucose-6-phosphate

H+: Ionizable Hydrogen

LD: *Longissimus dorsi*

LW: Large White

NAD+ and NADH: Nicotinamide Adenine Dinucleotide

NCBI: National Center for Biotechnology Information

PL: Piau-Large White

PP: Piau

qPCR: Real-time Polymerase Chain Reaction

QTL: Quantitative Trait Locus

RNA: Ribonucleic Acid

SECEX: Secretariat of Foreign Trade

SSC: *Sus scrofa*

Thr172: Phospho-*AMPK* alpha Antibody

ρs: Spearman correlation

SUMMARY

INTRODUCTION

Brazilian beef pig farming stands out as a sector of extreme relevance, particularly in economic terms, prioritizing large-scale production, quality, and a more sustainable approach to the use of inputs. Nonetheless, the increasing market demand, especially for premium cuts such as ham and loin, has driven an update in animal genetic improvement programs (Zhang *et al.,* 2019). Consequently, besides evaluating traditional productive traits such as feed conversion and efficiency, there is a growing consideration of meat characteristics, which can be comprehensively measured by parameters such as pH, water holding capacity (WHC), color, and intramuscular fat content (Fávero and Figueiredo, 2009; Zheng *et al.,* 2023).

Among these parameters, muscle pH has an intrinsic relationship with meat quality, as it directly influences these characteristics, impacting the final consumer experience (Alonso *et al.,* 2009). Initially, the pH in muscle tissue in vivo is close to neutrality (7.0), but during the conversion of muscle to meat, biochemical, energetic, and physical changes occur, resulting in alterations in this value (Matarneh, Scheffler and Gerard, 2023). These changes are due to the degradation of glucose via the anaerobic pathway, caused by insufficient or intermediate oxygenation due to the cessation of blood circulation at the time of slaughter, known as anaerobic glycolysis, in addition, to the fact that, during this process, hydrogen ions and lactate are produced and accumulate in muscle tissue.

This accumulation, combined with changes in the ATP/AMP ratio, leads to modifications in the activity of glycolytic enzymes, altering the pH and the rate of pH decline (Bai *et al.,* 2023). It is noteworthy that the glycolytic process should occur gradually, as an abrupt process results in pale, soft, and exudative (PSE) meat, while a very slow pH decline can lead to dark, firm, and dry (DFD) meat (Adzitey and Nurul, 2011).

Furthermore, besides glycolytic enzymes, the composition of muscle fiber also influences this decline. The development of skeletal muscle occurs in two stages: the first determines the potential for muscle mass in the animal's adult life, with the formation of prenatal muscle fibers and maturation, whereas the second is linked to the quantity and quality of meat, with the transformation of postnatal fibers (Lehka and Redowicz, 2020).

These postnatal fibers can be classified based on ATPase activity into slow oxidative muscle fibers (type I), fast oxidative muscle fibers (type IIA), fast glycolytic muscle fibers (type IIB), and intermediate muscle fibers (type IIX) (Brooke and Kaiser, 1970), which influences muscle glycogen content and post-mortem energy metabolism. Thus,

understanding the changes in pH, glycolytic enzymes, and muscle myofibrils during glycolysis is fundamental to ensuring high-quality production that meets consumer demands.

2. LITERATURE REVIEW

2.1 *Overview of Swine Farming in Brazil and Swine Breeds*

Brazilian swine production has increasing importance, demonstrating high competitiveness and relevance in the country's economy. Currently, Brazil ranks fourth globally, with 4,983 million tons produced, of which 22,48% are destined to export (SECEX; ABPA, 2022). Per capita consumption of pork is lower compared to beef and poultry protein sources. However, between 2012 and 2022, this consumption increased by 21%, reaching 18 kg/person (ABPA, 2022). These numbers can be attributed to improvements in the population's purchasing power, as well as intensive investment in the selection and genetic development of the species, providing consumers and industry with high-quality product (García-Gudiño *et al.,* 2021).

The origin of local breeds found in Brazilian territory and other Latin American countries descend from animals introduced by Portuguese and Spanish colonizers in the 16th century (de Lima *et al.,* 2018). Subsequently, crossbreeding and natural selection occurred according to environmental conditions, resulting in gradual modifications in reproductive and performance characteristics and the emergence of various local breeds such as Piau, Canastra, Monteiro, Moura, Caruncho, Pereira, and Nilo (Souza *et al.,* 2009; McManus *et al.,* 2010).

Among these local breeds, Piau is the most notable, being used in some breeding programs since 1939 with the aim of obtaining animals suitable for meat and fat production (Vianna, 1956; Gomes and D'Aulísio, 1980). It is characterized as a lard-type breed, with white or cream-colored coat with black or reddish spots, smooth and evenly distributed hair (Castro *et al.,* 2003). As a breed of the fat-type, adapted to tropical climates and resistant to diseases (Guimarães and Lopes, 2001; Mariante *et al.,* 2009; Sollero *et al.,* 2009; Silva *et al.,* 2019; Sprícigo *et al.,* 2019), it has low productive performance (Silva *et al.,* 2019) and high levels of subcutaneous and intramuscular fat (Serão *et al.,* 2011; Veroneze *et al.,* 2014).

However, changes in consumer preferences, particularly in the 1960s, led to an increase in the importation of foreign breeds with higher meat deposition capacity (Fávero and Figueiredo, 2009), resulting in the almost disappearance of local breeds from the Brazilian production system (Araújo *et al.,* 2020). Among the foreign breeds most used in the formation of commercial lineages for Brazilian production, Landrace, Large White, Duroc, and Pietran stand out (Yang *et al.,* 2017).

Large White is a typical European breed, known for its muscling and leanness (Briggs, 1969), as well as its prolificacy, originating from the northern region of England (Sarcinelli, Venturini and Silva, 2007). Morphologically, it has a concave profile, white coat, with excellent maternal ability, high prolificacy, reproductive precocity, as well as high carcass yield, excellent feed conversion, and good average daily weight gain (Lovatto, 1996). Another prominent breed in the Brazilian herd is Landrace. Originating from Denmark, it has a white coat and straight cephalic profile. They are animals with excellent reproductive capacity, prolific, precocious, with good maternal ability, and demonstrate good productive performance, with excellent ham yield (Lovatto, 1996).

The Duroc pig is the oldest breed, originating from United States and constituting a base for many mixed commercial lines, generally used as a terminal sire, due to its superior growth and meat quality, with high intramuscular fat content and pH (Choi *et al.,* 2014; Kim *et al.,* 2020). However, its reproductive performance and maternal ability are lower to the Landrace and Large White breeds (Nowak *et al.,* 2020). Phenotypically, it presents a reddish-brown color, broad structure, medium length, and muscular build with a sub-concave profile partially drooping, and a slight arching on the back. Finally, the Pietrain breed, originating from Belgium, resulting from the crossbreeding of Berkshire and Tamworth pigs. This breed was selected for muscularity and leanness, but Pietrain animals have relatively poor meat quality, due to the T allele of the RYR1 gene (Stratz *et al.,*2014).

2.2 ***Post-mortem metabolism and swine meat quality***

The first metabolic pathway to be elucidated was glycolysis, a nearly universal central pathway of glucose catabolism (Chandel, 2021). In other words, this molecule is degraded through a series of enzymatic reactions, divided into two phases, resulting in two molecules of pyruvate and free energy in the form of ATP and NADH (Nelson and Cox, 2022). This pyruvate is metabolized by three catabolic routes. The first involves oxidation into acetyl coenzyme-A, aerobically or under hypoxic conditions, either through alcoholic fermentation or lactic fermentation (Zangari *et al.,* 2020). In the latter process, pyruvate is reduced to lactate-by-lactate dehydrogenase to regenerate NAD+ and prevent the cessation of energy-generating reactions (Dhami *et al.,*2018).

The fermentative process provides ATP energy extraction without oxygen consumption and without variations in NADH or NAD+ concentrations. Moreover, this

mechanism is self-sufficient, independent of other energy pathways for electron acceptor regeneration (Nelson and Cox, 2022). Lactate formed by skeletal muscles can be recycled through transport via the bloodstream to the liver, where it is converted into glucose (Rui, 2014). However, during the slaughter process, after stunning and exsanguination, the muscle works to maintain energy homeostasis. Consequently, the phosphagen system is activated, with phosphocreatine degradation for AMP rephosphorylation into ATP through the enzyme creatine kinase (Wicks *et al.,*2022).

However, this system is unable to maintain homeostasis for a long period (Bendall, 1988). Therefore, progressively, muscular glycogen is anaerobically catabolized (Schiaffino and Reggiani, 2011), leading to lactic acid accumulation, increasing H+ ion concentration, and consequently acidifying the intracellular environment (Terlouw *et al.,* 2021). This process is initially rapid, stabilizing around 24 hours in swine species due to glycogen depletion, AMP deficiency, and/or inhibition of glycolytic enzymes due to low pH values, which at the end of 24 hours fall within the range of 5,50 to 5,70 (Astruc and Terlouw, 2023).

Given the economic importance of meat quality, swine breeding programs have included in their guidelines decisive properties such as color, water-holding capacity (WHC), marbling, intramuscular fat, tenderness, and pH, for which the rate and extent of decline during the *post-mortem* period influence overall quality determination (Noidad *et al.,* 2019). A rapid pH decline while the carcass temperature is still high results in a condition called PSE, when the meat appears pale, soft, and exudative due to sarcoplasmic and myofibrillar protein, resulting in texture integrity loss, discoloration, and impaired WHC (Honikel and Kim, 1986; Hughes *et al.,* 2014). In other words, it is an unpleasant product from both sensory perspectives, affecting tenderness due to moisture loss (Matarneh, Scheffler and Gerrard*,* 2023).

Initially, a high incidence of PSE meat was associated with swine stress due to a mutation in the halothane gene, resulting in increased calcium release from the sarcoplasmic reticulum due to the encoding of type 1 ryanodine receptor (RYR1) (O'Brien, 2002). However, the selection process eradicated this mutation in the commercial swine population (Petracci, Bianchi and Cavani, 2009). Nonetheless, metabolic stress before slaughter releases epinephrine, which induces glycogenolysis through the phosphorylase kinase-glycogen phosphorylase pathway, when protein kinase A phosphorylates RYR1 and increases calcium release, triggering responses similar to swine with the mutation (Andersson *et al.,* 2012).

2.3 ***Skeletal muscle – muscle fiber***

Skeletal muscle plays a crucial role in supporting and moving the body (Berridge *et al.,* 2018), composed of multinucleated cylindrical cells organized in bundles surrounded by muscle fibers (Zierath *et al.,* 2004). Its composition is divided into 74% water, 18% protein, 4 to 5% lipids, 1% carbohydrates, and 1% other substances such as vitamins, mainly from the B group, Zinc, Selenium, and Iron, which vary according to species, breed, and part of the animal carcass muscle. Muscle fibers are primarily composed of different types of contractile, cytoskeletal, sarcoplasmic, and regulatory proteins, distributed throughout highly organized myofibril areas that alternate with repeated structures known as sarcomeres (Choi and Kim, 2009), bounded by Z discs (Listrat *et al.,* 2016).

Histological and biochemical analyses conducted at the end of the 1960s were crucial to identify specific morphological, contractile, and metabolic properties for each of the numerous types of muscle fiber (Bergstrom and Hultman, 1973; Pette and Staron, 2000), resulting in a grouping based on metabolic enzyme profiles, protein isoforms, and structural and contractile properties (Schiaffino and Reggiani, 1996; Bottinelli and Reggiani, 2000).

Each fiber represents a syncytium formed during myogenesis, in which function depends on the functionality of the nervous system to initiate contraction and involves intrinsic mechanisms to direct calcium ion movement in the sarcoplasmic reticulum during the contraction-relaxation cycle (Berridge *et al.,* 2018). The metabolic properties of each fiber group are crucial for thermoregulation, maintenance of basal energy, as well as for the storage of carbohydrates and amino acids, processes who utilize ATP through glucose conversion and degradation of muscle glycogen, or through lipid degradation, ketone bodies, or even free and volatile fatty acids (Feng *et al.,* 2024). Therefore, based on these characteristics, muscle tissue constitutes an important energy reserve and plays a crucial role in the pre- and post-mortem periods, mainly influencing the conversion of muscle into meat (Ryu and Kim, 2006; Matarneh, Scheffler and Gerrard, 2023).

In 1970, Guth and Samaha developed the classification of fibers through histochemical processes, assessing the differential sensitivity of ATPase to myosin isoforms at acidic and alkaline pH levels, thus distinguishing acid-resistant fibers, type II with fast contraction, from acid-sensitive slow-twitch fibers, type I, due to ATPase inactivation and whitening acquired

by fast fibers when placed in acidic conditions, while type I fibers maintained enzymatic activity and acquired a black coloration. Through different acidic and basic pre-incubation methods, using myosin activity labeling, fibers were also classified into subtypes: IIA, IIB, and IIX (Staron and Pette, 1993; Staron *et al.,* 1999; Picard and Gagaoua, 2020).

Since last decade, immunohistochemical techniques are using polyclonal and monoclonal antibodies directed to MHC. These techniques allow a more efficient differentiation between muscle fibers containing only a single *MHC I*soform, from those with multiple isoforms (Meunier *et al.,* 2010; Scheffler *et al.,* 2018; Astruc *et al.,* 2023), which contributed to the discovery type IIB fiber, which is only expressed in small mammals such as rats, rabbits and pigs (Kohn *et al.,* 2007).

The existing diversity of *MHC I*soforms results from gene regulation in two mechanisms, the first being qualitative, in which isoforms can be derived from the same gene through alternative splicing, or different genes from the same family. The second mechanism is quantitative, when several genes can be up or down regulated independently, thus, the proportion between the products of these genes is modified and new functional or structural characteristics appear (Bottinelli and Reggiani, 2000).

The classification of muscle fibers by contraction speed and metabolic properties is the reason why *MHC I*soforms have been directly involved as drivers of the initial *post-mortem* metabolism rate, including the pH decline, as there is a correlation with the decline of muscle glycogen at the time of slaughter. For example, for fibers with lower glycogen content, such as IIX and IIB, the pH decay rate occurs more slowly, as well as the accumulation of lactic acid (Astruc and Venien, 2017). In summary, the greater pH decline can be associated with muscles that have a higher concentration of fast glycolytic fibers (Gagaoua *et al.,* 2017).

Moreover, the glycolytic rate influences myofibrillar proteins, such as actin, troponin, and myosin, and some metabolic proteins, especially glycolytic enzymes found in the sarcoplasm. Such proteins when altered during the *post-mortem* period, cause changes in the final quality of the meat, affecting tenderness, juiciness, flavor, and color (Picard and Gagaoua, 2020).

The designation of slow-twitch fibers is due to the wider Z bands, thus having less area for ATP resynthesis, which predominantly occurs through mitochondrial oxidative phosphorylation, with electron transport chain intermediates after glucose and fatty acid

oxidation in the citric acid cycle. In contrast, fast-twitch fibers have a smaller width, resulting in an intrinsic contraction speed and fiber shortening three times faster, with ATP resynthesis occurring mainly through anaerobic glycolysis and glycogenolysis (Berridge *et al.,* 2018). Additionally, type IIb fibers present a well-developed sarcoplasmic reticulum (SR) system and tubular invaginations of the plasma membrane (T-tubules), further contributing to their contractile speed (Bottinelli *et al.,* 1994; Stienen *et al.,* 1996), as shown in Table 1 below.

Table 1. Physiological and biochemical characteristics of muscle fiber types

Fibers	**I**	**IIA**	**IIX**	**IIB**
Gene	***MYH7***	***MYH2***	***MYH1***	***MYH4***
Contraction velocity	+	+++	++++	+++++
ATPase activity	+	+++	++++	+++++
SDH activity	+++++	++++	++	+
Oxidative metabolism	+++++	++++	++	+
Glycolytic metabolism	+	++++	++++	+++++
Glycogen	+	+++	+++	++++
Phosphocreatine	+	+++++	+++++	+++++
Memory capacity	+	++++	+++++	+++++
Z-band width	+++++	+++	+++	+
Cross-sectional area	+	++	++++	+++++
Red color (Myoglobin)	+++++	+++	++	+

+ very low; ++ low; +++ moderate; ++++ high; +++++ very high. Source: Picard e Gagaoua, 2020 (adapted)

The types of fibers are influenced by hormones (Florini *et al.,* 1996; Karlsson *et al.,* 1999, Choi and Kim, 2009), such as thyroid hormones that play an important role during muscle development and maturation (Ianuzzo *et al.,* 1977), when low levels inhibit or delay the appearance of fast-twitch muscle fibers, while elevated levels accelerate the transition from developmental to fast-twitch fibers, including Myosin isoforms (Butler-Browne and Whalen, 1984; Adams *et al.,* 1999). β-adrenergic receptor agonists contribute to increasing fiber area and converting type IIA fibers to IIB (Oksbjerg *et al.,* 1995). In the case of endocrine factors, serum concentrations of insulin-like growth factor I (IGF-I) negatively influence the percentage of type I fibers, while serum epidermal growth factor acts positively (Ryu *et al.,* 2008).

2.4 ***Transcripts analyzed in this work***

2.4.1 ***HK2***

Hexokinase initiates glucose-dependent pathways by promoting a concentration gradient that facilitates glucose entry (Bell *et al.,* 1993; Mueckler, 1994). It consists of a family with four isoforms: HK1, HK2, HK3, and HK4, with HK1 and HK2 being the most abundant. HK1 is primarily expressed in the brain and red blood cells (Lowry and Passonneau, 1964; Purich and Fromm, 1971), while HK2 is found mainly in insulin-sensitive tissues such as adipocytes and adult skeletal and cardiac muscle (Mandarino *et al.,* 1996).

Additionally, HK2, a gene located on SSC3 of Sus scrofa, is associated with glucose metabolism and glycolytic regulation (Wei *et al.,* 2016; Miller *et al.,* 2019) due to its crucial role as the first rate-limiting enzyme of glycolysis, catalyzing the conversion of glucose to glucose-6-phosphate with ATP consumption (Fang *et al.,* 2012). The combination with the mitochondrial outer membrane through the voltage-dependent anion channel of the surface protein results in the acceleration of the glycolysis rate (Li *et al.,* 2017).

2.4.2 ***PFKM***

The glycolytic enzyme phosphofructokinase is a key regulatory enzyme in glycolysis, representing an important control point in glucose metabolism. Mammalian PFK is a tetramer composed of three subunits, PFKL expressed in the liver, PFKP in plasma and *PFKM* primarily expressed in skeletal muscle tissue (Dunaway *et al.,* 1988). Muscular phosphofructokinase (*PFKM*) is a key regulatory enzyme that catalyzes the irreversible conversion of fructose-6-phosphate to fructose-1,6-bisphosphate, a step that regulates various biological processes such as glucose metabolism and muscle maintenance (Raben *et al.,* 1995; Wegener *et al.,* 2002). Additionally, *PFKM* is encoding pyruvate kinase, an enzyme responsible for the transformation of phosphoenolpyruvate into pyruvate (Fontanesi *et al.,* 2003; Sieczkowska *et al.,* 2010).

Located at SSC5, *PFKM* is the main regulator of glycolytic efficiency (Tang *et al.,* 2012). Its expression level influences the quality of pork, as it strongly correlates with marbling and intramuscular fat content (England *et al.,* 2014; Zequan *et al.,* 2021) and coincides with regions of QTL for fatty acid composition (Pertek, 2011) and average daily gain (Wang *et al.,* 2014). Furthermore, this enzyme reversibly associates with different cellular components, such as actin filaments, microtubules and the integral membrane anion

transporter (Beitner, 1993; Assouline-Cohen and Beitner, 1999). This process is dynamically regulated by association with caveolin-3 proteins in differentiated myotubes and regulated by extracellular glucose and intracellular metabolites (Scherer and Lisanti, 1997).

PFKM is one of the few enzymes that can be inhibited by the high concentrations of its own substrate, ATP (Costa Leite *et al.,* 2007; Zancan *et al.,* 2007; Zancan *et al.,* 2008). Additionally, the end product of glycolysis, pyruvate, enters the Krebs cycle, where it is converted into citrate, which also acts as a negative feedback inhibitor of glycolysis. This phenomenon also observed in lactate accumulation during the conversion of pyruvate to lactate in anaerobic processes (Costa Leite *et al.,* 2007; Marinho-Carvalho *et al.,* 2009).

Moreover, post-translational modifications of *PFKM* affect various functions. For instance, N-glycosylation plays an important role in development, body growth and organ formation (Zielinska *et al.,* 2012). Similarly, the modification of β-N-acetylglucosamine linked to oxygen regulates glycolytic metabolism (Yi *et al.,* 2012).

2.4.3 ***AMPK***

In mammals, the adenosine monophosphate-activated protein kinase (*AMPK)* exists as a heterotrimeric enzyme complex formed between catalytic (alpha) and regulatory (beta and gamma) subunits, along with glycogen. There are two known alpha subunit isoforms, designated alpha-1 and alpha-2, two beta subunit isoforms (beta-1 and beta-2), and three gamma subunit isoforms (gamma-1, gamma-2, and gamma-3) (Woods *et al.,* 1996), encoded by the genes *PRKAA1*, *PRKAA2*, *PRKAB1*, *PRKAB2*, *PRKAG1*, *PRKAG2*, and *PRKAG3*, respectively (Hardie*,* 2011).

AMPK serves as a metabolic regulator of various intracellular pathways, including glucose uptake and glycogen synthesis, playing a crucial role in skeletal muscle (Woods *et al.,* 1996; Hardie and Carling, 1997; Sambandam and Lopaschuk, 2003; Carling, 2004). Additionally, it influences fatty acid β-oxidation, controlling metabolism through transcription and having direct effects on metabolic enzymes (Winder, 2001; Mihaylova and Shaw, 2011).

Activation of *AMPK* occurs when ATP levels are depleted, specifically due to an increase in the AMP:ATP ratio, which adjusts anabolic and catabolic rates, i.e., consumption and generation of ATP, respectively (Hardie and Carling, 1997; Kemp *et al.,* 1999; Hardie*,*

2011). Consequently, it functions as a sensor of the cell's energy state (Carling, 2004; Winder *et al.,* 2001). The ratio increase can result from oxygen supply restriction or the presence of oxidative phosphorylation inhibitors, which activate *AMPK* due to increased phosphorylation at Thr172 within the alpha subunit (phospho-*AMPKα*), thus activating glycolysis (Sambandam and Lopaschuk, 2003; Hardie*,* 2011).

In various tissues, such as liver, adipose tissue, and skeletal muscle, *AMPK* phosphorylates and inactivates several key biosynthetic pathway enzymes, such as glycogen synthase (Young, Radda, and Leighton, 1996), thereby conserving ATP. Additionally, it is believed to mediate the recruitment of glucose transporters (GLUT4) (Kurth-Kraczek *et al.,* 1999; Russell *et al.,* 1999). Glycolytic increase may occur through phosphorylation of phosphofructokinase-2 (*PFK-2*), catalyzing the production of fructose-2,6-bisphosphate, an allosteric activator of *PFK-1* (Marsin *et al.,* 2000). This demonstrates a fundamental role in post-mortem glycolysis, critically regulating the incidence of pale, soft, and exudative meat (Shen *et al.,* 2006). Moreover, it has long-term effects, altering gene expression (Foretz *et al.,* 1998; Yang *et al.,* 2001).

Among these subunits, α2-*AMPK* and γ3-*AMPK* have significant effects on pork meat quality. The *PRKAA2* gene regulates glucose metabolism, lipid metabolism, and protein synthesis in peripheral tissues, playing an important role in skeletal muscle gene expression differences (Viollet *et al.,* 2003; Pan *et al.,* 2011), and is related to the regulation of energy intake and animal body weight (Wu *et al.,* 2018). The γ3 subunit gene activated by protein kinase (*PRKAG3*) is particularly interesting in muscle physiology because it is the only *AMPK* subunit specifically expressed in skeletal muscle, showing metabolic plasticity in fast and glycolytic fibers (Mahlapuu *et al.,* 2004; Essén-Gustavsson *et al.,* 2011).

This gene, PRKAG3, located on SSC15, has economically important missense mutations with additive effects on glycogen content in meat quality traits of various pig breeds, such as pH values, water-holding capacity (WHC), and color (Milan *et al.,* 2000; Ciobanu *et al.,* 2001). Additionally, it participates in mitochondrial biogenesis, fatty acid uptake, and muscle oxidative capacity (Nilsson *et al.,* 2006; Crawford *et al.,* 2010).

2.4.4 ***PPARGC1A***

The gene peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A* or *PGC1-α*), located on SSC8 dos *Sus scrofa*, is aligned with energy metabolism, playing a crucial role in muscle and adipose tissue, and liver metabolism. This gene serves as an important transcription factor in controlling genes related to slow-twitch muscle fiber specification and oxidative metabolism (Sevane *et al.,* 2013). It activates a complex pathway of mitochondrial biogenesis while simultaneously modulating biological processes associated with increased tissue oxidative capacity, contributing to glucose metabolism (Lin, Handschin, and Spiegelman, 2005). In skeletal muscle, it controls multiple pathways, acting as a sensitive "rheostat" to neuromuscular stimuli and resulting contractile activity (Norrbom *et al.,* 2004). Additionally, it is capable of coupling metabolic and contractile processes of muscle fibers, converting fast-twitch fibers into slower, more oxidative fibers (Lin, Handschin, and Spiegelman, 2005).

Its potent effect on cellular energy metabolism requires rigorous control, which theoretically can be achieved at the transcriptional level, responding to nutritional and environmental stimuli (Puigserver *et al.,* 1998; Lin, Handschin, and Spiegelman, 2005). Moreover, regulation can also occur post-transcriptionally, with the displacement of the myb p160 binding protein (p160MBP), a strong transcriptional suppressor (Fan *et al.,* 2004). Regulation can also occur through *AMPK*, which, in cases of low energy, induces *PGC-1α*, resulting in increased energy levels, consequently elevating oxidative metabolism (Zong *et al.,* 2002).

3. **HYPOTHESES**

There are significant variations in muscle gene expression and the rate of muscle pH decline associated with glycolytic metabolism among different swine genetic groups, Piau, Large White, and crosses between Piau-Large White and Duroc-Large White.

4. **OBJECTIVES**

4.1 ***General***

To investigate the existence of distinct gene expressions that may explain glycolytic metabolism through muscle pH analysis, and to characterize this expression by evaluating the influence of local breeds in crosses compared to commercial lines.

4.2 ***Specific***

To measure muscle pH in purebred and crossbred animals at different post-slaughter times;

To evaluate and analyze the rate of muscle pH decay according to genetic group;

To compare gene expression among genetic groups: Piau, Large White, and the Piau-Large White and Duroc-Large White crosses;

To investigate differential gene expression related to animal glycolytic metabolism.

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CHAPTER 1

INTRODUCTION

In the animal production system, profitability is directly linked to animal performance characteristics. However, traits related to meat quality play a crucial role in the productive system as well as in consumer satisfaction (Marzoque *et al.,* 2020), making the assessment of pork quality highly important.

In evaluating meat quality, the growth and muscle development of the animals play an essential role. This process is complex and includes the formation and development of muscle fibers, as well as muscle regeneration during the adult stage (Pan *et al.,* 2023). In skeletal muscle, there are four types of fibers, which differ in proportion, metabolism, and physiological function, resulting in changes in pork quality (Wimmers *et al.,* 2007; Guo *et al.,* 2012).

Moreover, the differential expression of genes related to animal glycolytic metabolism is directly associated with meat quality. Genes related to glycolytic enzymes, such as Hexokinase-2 (*HK2*) and Phosphofructokinase (*PFK*), as well as the adenosine monophosphate-activated protein kinase (*AMPK*) gene, play an important role in meat quality attributes as they influence muscle glycogen levels and the type of muscle fiber in pigs (McGresham *et al.,* 2014; Matarneh*,* Scheffler and Gerrard, 2023).

Among the meat quality attributes, the muscle hydrogen potential (pH) stands out, which varies between pig breeds due to sex, age, fat thickness, and muscle type (oxidative or glycolytic) of the animal (Terlouw *et al.,* 2021; Kent *et al.,* 2024). These variations become even more evident when comparing unselected animal with those of exceptional genetics (Zhang *et al.,* 2016). Therefore, simultaneously evaluating local and commercial breeds provides a better understanding of these differences, enabling a more precise choice of genotypes for specific traits.

An example is the Piau breed (PP), a Brazilian local breed characterized by lower slaughter weight and low reproductive rates but with excellent intramuscular fat deposition, conferring good quality attributes (Serão *et al.,* 2011; Veroneze *et al.,* 2014). In contrast,

commercial breeds such as Large White (LW) and Duroc (DL) are known for their reproductive and productive superiority, with high prolificacy and precocity, good feed efficiency, and higher carcass yield (Wang *et al.,* 2022). However, due to intense genetic selection for high productive performance and higher lean tissue content, the meat quality of these breeds has fallen short of expectations (Saikia *et al.,* 2024).

Given that local breeds tend to have better meat quality indices, the question arises to what extent genetics influence glycolytic metabolism, affecting pH values, muscle behavior, and glycolytic enzymes. Therefore, this study aims to investigate the existence of distinct gene expressions that can explain glycolytic metabolism through muscle pH analysis and characterize this expression by evaluating the influence of local breeds in crossbreeding compared to commercial lines.

2. MATERIALS AND METHODS

All methods involving animals' manipulation followed the ethical principles of animal research (CONCEA, 2016) and were approved by the Ethics Committee on the Use of Production Animals (CEUAP) of the Universidade Federal de Viçosa, protocol Nº. 014/2022.

2.1 *Animals*

The experimentation took place at the Teaching, Research, and Extension Unit in Swine Breeding (UEPE-MS) at Universidade Federal de Viçosa (UFV). Thirty-nine castrated males with an initial age of 70 days, originating from four genetic groups: Piau (PP), Large White (LW), and the crosses Piau x Large White (PL) and Duroc x Large White (DL).

During the rearing and finishing phases, animals standardized according to age, were housed in individual stalls and subjected to similar nutrition and management conditions, as well as having access to *ad libitum* water and feed. Diets were formulated to meet or exceed nutritional needs based on Rostagno *et al*. (2017) recommendations, considering animals of commercial lineage. Following a four-phase program according to the animal's growth stage: growth 1, growth 2, finishing 1, and finishing 2. At 70 days and 170 days, the average weight of the animals in each genetic group was measured using an electronic scale, followed by ANOVA and Tukey test at 5% significance, as presented in Table 2.

Table 2. Body weight of the four genetic groups at 70 and 170 days.

	Age[2]		Weight[3]	
Genetics groups[1]	**Initial**	**Final**	**Initial weight**	**Final weight**
PP	70	170	17.37 ± 1.72[d]	81.10 ± 6.72[d]
LW			29.76 ± 3.32[b]	130. 56 ± 8.31[b]
DL			31.85 ± 2.03[a]	150.51 ± 8.74[a]
PL			27.13 ± 1.62[c]	115.89 ± 6.72[c]

[1]PP: Piau; LW: Large White; DL: Duroc-Large White; PL: Piau-Large White;
[2]Age in days;
[3]Means with the same letter in the same column are not significantly different (*p-value* <0.05).

2.2 *Measurement of carcass pH*

The 39 Animals were slaughtered at 177 days of age (±2.3 days) and had an average weight of 125.46 kilograms (±24.61 kg), after 16 hours of fasting, following the animal welfare standards required by the Sanitary and Industrial Inspection Regulation of Animal Origin Protocols - RIISPOA (Brazil, 2017), with electrical stunning and subsequent jugular section.

The pH recording and muscle temperature were performed at eleven different times: at slaughter being time zero (pH0), 45 minutes after slaughter (pH0.75), 1, 2, 3, 4, 5, 6, 7, 8, and 24 hours after slaughter (pH24). The pH values were obtained using a pH meter (Hanna-DIGIMED DM-20 model) coupled with a penetration probe (DIGIMED, DME-CV1), inserted into the center of the *Longissimus dorsi* (LD) in the left half carcass, between the 12th and 13th thoracic vertebrae.

The pH data was analyzed using the repeated measures model over time, including muscle temperature as a covariate and time, genetic group, and the time-genetic group interaction as fixed effects. Various residual variance structures were tested, such as to identify the model with the best fit according to corrected AIC values (Akaike Information Criterion). The analyses were conducted using Linear and Nonlinear Mixed Effects Models, *nlme* in the R software version 4.3.1. Subsequently, an ANOVA was performed followed by the comparison of treatment means using the Tukey test (*p-value* <0.05).

The analysis of muscle pH decay was conducted using R software version 4.3.1, employing the *nls2* package with data adjustment according to the following exponential model proposed by Bruce, Scott and Thompson (2001), where muscle pH was expressed as a function of time (hours post-stunning):

$$\textbf{Measured } pH_t = pH_{final} + ae^{-kt} \textbf{ (1)}$$

where:

pH_t is the muscle pH at time t (hours after stunning);

pH_{final} is the final or infinite pH of the muscle (at 24 hours after stunning);

a is the estimate of the extent of pH decline;

k is the rate constant of the pH decline.

The model Eq.1 was used to obtain values for *a* and *k* for each genetic group. The graphical representation was performed using the *ggplot2* package.

2.3 *RNA collection and extraction from swine muscle*

Immediately after exsanguination, at time zero (pH0), samples of the LD were stored in pre-labeled 2 ml cryotubes. These were frozen in liquid nitrogen and subsequently stored at -80°C until the second stage, which took place in the Animal Biotechnology Laboratory (LABTEC) of the Department of Animal Science at the Universidade Federal de Viçosa (DZO/UFV).

Total RNA was extracted from 0.5 mg of frozen LD muscle samples, at time zero of pH measurement, immediately after slaughter. Initially, the samples underwent mechanical lysis using a Turrax®, aiming to ensure better extraction process results. Then phenol: chloroform extraction protocol, using Trizol® (Ambion, Life Technologies, Carlsbad, CA) was used, according to the manufacturer's instructions, to extract total RNA from the samples. Sample integrity was verified by electrophoresis on a 0.8% agarose gel stained with ethidium bromide.

The RNA sample concentration was determined by the Qubit® RNA BR Assay kit (Invitrogen, Carlsbad, CA), following the manufacturer's recommendations, and analyzed by the Fluorometer, Qubit® 3.0 (Thermo Scientific, Wilmington, DE, USA). Purity was determined by calculating the ratio of absorbance at 260 nm and the absorbance at 280 nm (A260/A280) using the NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). After this step, twenty-four samples were chosen according to the RNA concentration, with 6 samples per genetic group.

2.4 *RT-qPCR analysis*

For qPCR analysis, 1µg of RNA was treated with DNase I® kit (Invitrogen, Carlsbad, CA), following manufacturer's recommendations. Then, the treated RNA underwent reverse transcription (RT) using GoScript™ Reverse Transcription Mix, OligodT kit (Promega, Madison, WI, USA), following the manufacturer's recommendations. cDNA quantification was performed using Qubit ssDNA Assay kit, in the Qubit 3.0 Fluorometer, and stored at -20°C.

Primers for the transcripts of each selected gene (Table 3) were designed using NCBI's PrimerBlast software, with subsequent efficiency analysis by NeTPrimer (http://www.premierbiosoft.com/NetPrimer/AnalyzePrimer.jsp). The efficiency test was

performed using GoTaq qPCR Master Mix kit (Promega Biosciences, USA), employing three final concentrations, with a final reaction volume of 10µl, containing 1xMaster Mix, with primer concentrations at 100, 200, or 400 nM, and the amount of cDNA used varying between 5, 15, or 45 ng. The final primer concentrations were chosen according to the from the slope (S) of standard curves: $E = 10^{-1/S} - 1$.

Real-time PCR reactions were performed at CFX96 Touch Real-Time PCR Detection System thermocycler (Bio-Rad, Foster City, CA, USA) using the GoTaq® qPCR Master Mix kit (Promega, Madison, WI, USA). Amplification conditions for all systems were: 95°C for two minutes, 40 cycles of denaturation at 95°C for 15 seconds, and extension at 60°C for 1 minute. At the end of the amplification cycles, melting curve analysis was performed for each system.

The values of relative expression were calculated by the comparative cycle threshold method, $2^{-\Delta\Delta CT}$, described by Livak and Schmittgen (2001), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript based on *Sus scrofa* species was used as the endogenous control. Subsequently, an analysis of variance (ANOVA) and Tukey test were performed at a 5% significance level, using the equation:

$$\mathbf{Y_{gik} = \mu + TG_{gi} + \beta 1_i * PF_{ik} + P_{ik} + e_{gik}}$$

where:

µ is the overall mean;

TG_{gi} is the normalized Ct obtained by $2^{-\Delta\Delta CT}$ for gene g, genetic group i, of animal k;

$\beta 1_{i,}$ the covariate weight;

PF_{ik} is the weight on the day of slaughter (final) in genetic group i and animal k;

P is the plate used for genetic group i and animal k;

e_{gik} residual error ~ N (0, σ^2)

Table 3. Sequence of primers selected for the qPCR of the studied transcripts.

Gene	Primer Sequence (5'-3`)	NCBI Gene ID	Efficiency
HK2	F: CGCCGTGGTGGACAAAATAC R: CTCCAGGAAGGACACGTCAC	494561	94.00
PFKM	F: AACTGTGAAGAGGGGCTTGG R: GCCTTCGCACCCATCTTAGT	733601	95.00
PPARGC1A	F: AACCCACAGAGACCCGAAAC R: CCCTTGGGGTCATTTGGTGA	397013	95.00
PRKAA1	F: TGGCTCACCCAACTATGCTG R: ACCTCTGGACCAGCATACAAC	100145903	93.50
PRKAA2	F: GGGTCTCCAAATTACGCAGC R: TGCTGCGTAATTTGGAGACC	397504	95.00
PRKAG3	F: GCTGCCTGTGGTCAACGAAA R: CTCAGGGCTTCTCCCACATTC	397149	98.50
MHC I	F: CACACCTGTTGAGAAGGGCAA R: GTGCATGACCAGAGGATTGTC	396860	89.50
MHC IIA	F: GGCTCAAACTGGTGAAGCA R: GATGCAGCGCACAAAGTGAG	397256	89.50
MHC IIB	F: CCTCTTTGCTGAGAGACAGAGTTC R: TCCTCAGGTTGGTCATCAGC	100144306	94.00
MHC IIX	F: CAGGTGCTGATGCAGAGGC R: GTGCTCCTCAGGTTGGTCAT	100125538	93.00

HK2: Hexokinase-2; *PFKM*: Muscle-type phosphofructokinase; *PPARGC1A*: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; *PRKAA1*: AMP-activated protein kinase alpha-1; *PRKAA2*: AMP-activated protein kinase alpha-2; *PRKAG3*: AMP-activated protein kinase gamma-3 subunit. Isoforms of myosin heavy chain: *MHC I*: Myosin-7; *MHC IIA*: Myosin 2; *MHC IIB*: Myosin-1; *MHC IIX*: Myosin-4

2.5 *Gene ontology analysis and KEGG pathways*

Gene ontology (GO) analyses were conducted by STRING program (Jensen *et al.,* 2009) version 12.0 (https://string-db.org/), with *Sus scrofa* as the base organism (NCBI, reference genome Sscrofa11.1). This process involved obtaining representations of biological processes, molecular function, and cellular components, as well as the existence of gene pathways, where network enrichment was assessed according to strength, measured by the logarithmic function base 10 and through the false discovery rate (FDR) value. Additionally,

*p-value*s were corrected using the Benjamini-Hochberg procedure to decrease FDR, with a *p-value* of 5%

2.6 ***Correlation of the analyzed transcripts***

All groups were tested for normality before pH value analysis using the Shapiro-Wilk test and data dispersion assessment. Global correlation among different variables was conducted using the Spearman correlation test, as the data did not meet normal distribution criteria. This analysis aimed to investigate the correlation between genes and between genes and muscle pH values. For this purpose, R software version 4.3.1 was utilized with a confidence level of 95%, while significance was set at 5%. Heatmaps were generated using the *ggplot2* and *corrplot* packages in R.

3. **RESULTS**

3.1 ***pH***

In this study, significant pH variations among different swine breeds and genetic crosses were investigated. The pH is important for meat conservation and technological properties. Its variation affects the final meat quality, influencing consumer experience. Initially, by comparing means between the genetic groups, the local breed differed significantly ($p<0.05$) from the LW breed at measurement times 0, 0.75, 1, 3, 4, 5, 7, 8, and 24 hours (Table 4). Compared to the Duroc-Large White crossbreed, the results showed significance at 4-, 5- and 6-hours post-slaughter, while regarding the PL group, there was a statistical difference at 0.75-, 3-, 4- and 5-hour post-exsanguination. The LW group differed significantly from the DL group at 0- and 1-hour post-slaughter, as detailed in Table 4. Furthermore, analyzing the overall mean for the four genetic groups, it is noticeable that PP exhibited a higher pH value mean, whereas the Large White breed showed the lowest overall mean in the eleven muscle pH measurement times (Table 5). Regarding the assessment of significant overall differences among the genetic groups, it is noted that PP statistically differs from LW, DL and PL. Among the commercial breeds, LW and DL, a statistical difference ($p<0,05$) was also verified. Comparing the PL with the LW and DL, did not show significant differences ($p>0.05$) (Table 5).

Table 4. Descriptive statistics of muscle pH in the four analyzed genetic groups.

Measurement	Muscle pH*			
	PP	**LW**	**DL**	**PL**
pH_0[1]	6.64 ± 0.26[a]	6.16 ± 0.36[b]	6.48 ± 0.21[a]	6.28 ± 0.12[ab]
$pH_{0.75}$[2]	6.37 ± 0.19[a]	6.02 ± 0.24[b]	6.22 ± 0.23[ab]	6.05 ± 0.24[b]
pH_1	6.15 ± 0.19[a]	5.79 ± 0.22[b]	6.10 ± 0.31[a]	5.88 ± 0.23[ab]
pH_2	5.96 ± 0.20[a]	5.70 ± 0.22[a]	5.85 ± 0.35[a]	5.69 ± 0.29[a]
pH_3	5.98 ± 0.36[a]	5.57 ± 0.13[b]	5.80 ± 0.29[ab]	5.68 ± 0.29[b]
pH_4	6.10 ± 0.34[a]	5.61 ± 0.12[b]	5.73 ± 0.19[b]	5.75± 0.43[b]
pH_5	6.01 ± 0.33[a]	5.64 ± 0.19[b]	5.70 ± 0.16[b]	5.68 ± 0.21[b]
pH_6	6.01 ± 0.28[a]	5.72 ± 0.40[ab]	5.71 ± 0.20[b]	5.72 ± 0.25[ab]
pH_7	5.97 ± 0.20[a]	5.61 ± 0.12[b]	5.71 ± 0.16[ab]	5.67 ± 0.23[ab]
pH_8	5.96 ± 0.19[a]	5.63 ±0.12[b]	5.72 ± 0.13[ab]	5.71 ± 0.18[ab]
pH_{24}[3]	6.00 ± 0.18[a]	5.64 ± 0.14[b]	5.77 ± 0.27[ab]	5.73 ± 0.28[ab]

*Values followed by the same letter in the same row do not differ significantly by the Tukey test (p<0.05).
PP: Piau; LW: Large White; DL: Duroc-Large White; PL: Piau-Large White;
pH measured in 1, 2, 3, 4, 5, 6, 7 and 8 hours after stunning
[1] Measurements taken *post-mortem*.
[2] Measurements taken after 0.75 hours (45 minutes) of stunning.
[3] Measurements taken approximately 24 hours after stunning.

Table 5. Mean and standard deviation of muscle pH according to genetic group.

Genetics Group	Mean*	Median	Minimum	Maximum	CV[1]
PP	6.11 ± 0.31[a]	6.09	5.60	6.95	5.05
LW	5.74 ± 0.27[c]	5.65	5.33	6.74	4.79
DL	5.89 ± 0. 33[b]	5.80	5.38	6.86	5.66
PL	5.80 ± 0.31[bc]	5.74	5.24	6.98	5.29

*Means with the same letter in the same column are not significantly different (*p-value* <0.05).
PP: Piau; LW: Large White; DL: Duroc-Large White; PL: Piau-Large White.
[1]Coefficient of variation in percentage

In further evaluation of muscle pH, it was observed that as time progresses, especially up to three hours post-slaughter, there was for a decline in for pH values in all groups (Figure 1). Moreover, the DL group demonstrated a more pronounced decline in pH values initially, reaching stability near the rigor mortis value of the species.

The PP group exhibited an initial decline, resulting in the highest pH value at the end of the 24-hour analysis (Figure 1). However, upon examining the decay curve, similarities with the Large White breed are observed, with stability in the hours following slaughter. Nevertheless, this group, PL, shows higher muscle pH values, especially with a higher final pH value (pH24), possibly due to the presence of the Piau breed in its cross (Figure 1).

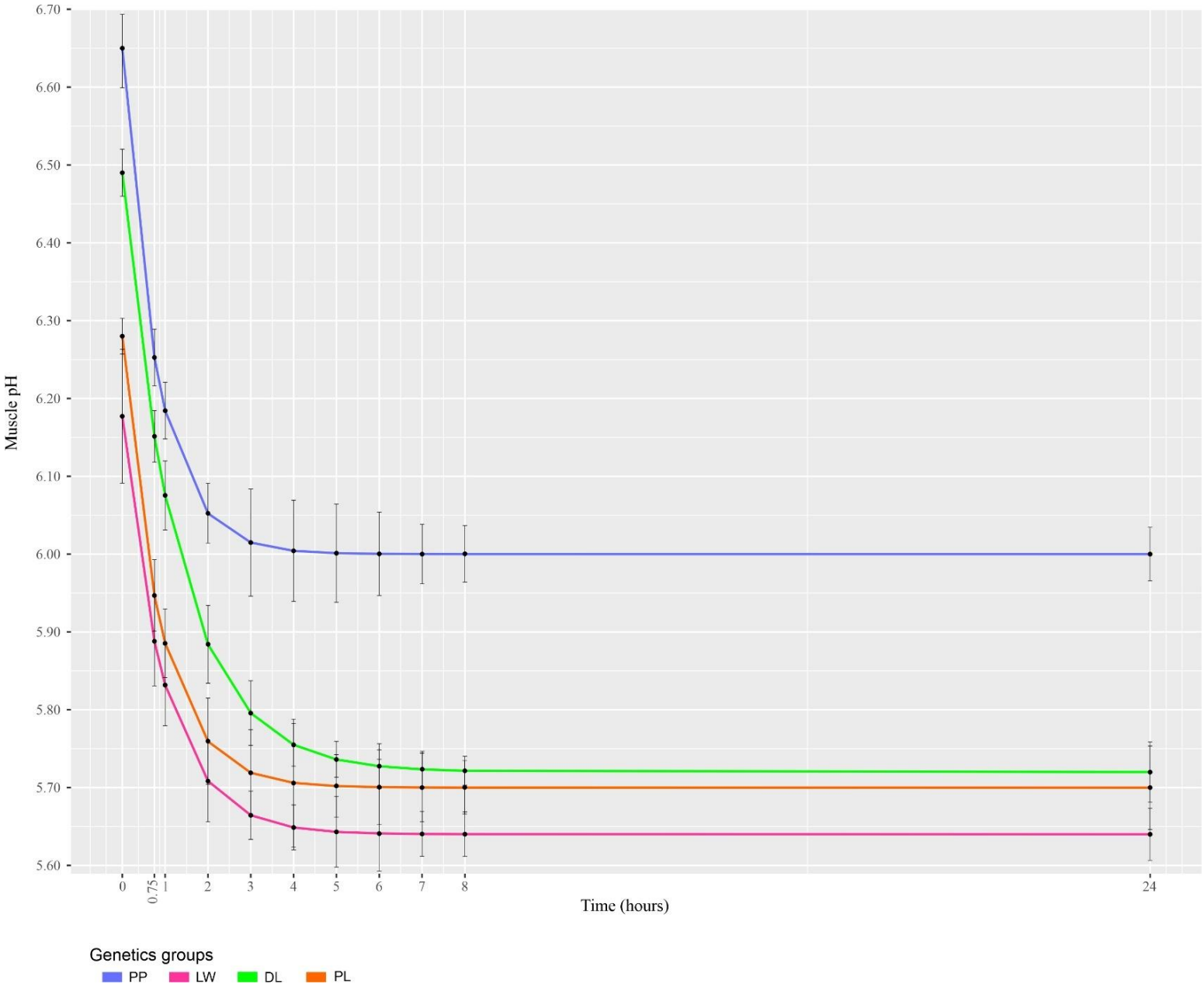

Figure 1. Decay curve of adjusted muscle pH in different genetic groups.

The error bar in the figure represents the standard deviation (SD). PP, Piau; LW, Large White; DL, Duroc-Large White and PL, Piau-Large White.

3.2 ***Transcripts expression analysis***

The gene expression analysis across different swine genetic groups, encompassing ten distinct genes, showed significant differences among one or more genetic groups, as can be seen in Figure 2.

The glycolytic pathway genes, *HK2* e *PFKM* exhibited higher relative expression in commercial breeds. In contrast, the catalytic subunits *PRKAA1* e *PRKAA2* showed higher values in the commercial breeds, in the DL cross and in the LW breed. Meanwhile, the regulatory subunit *PRKAG3* demonstrated greater expression in the DL and LW groups. The *PGC1-α* gene, which plays an important role in glycolysis metabolism and has a negative correlation with IMF content in pigs, showed higher relative expression in the PP and PL compared to the LW and DL groups. Lastly, the myosin isoforms displayed a pattern where the local breed (PP) and its crossbreed (PL) had higher relative expression of oxidative myosin isoforms, *MHC I* and *MHC IIA*, while the glycolytic isoforms, *MHC IIB* and *MHC IIX*, were more expressed in the DL and LW groups.

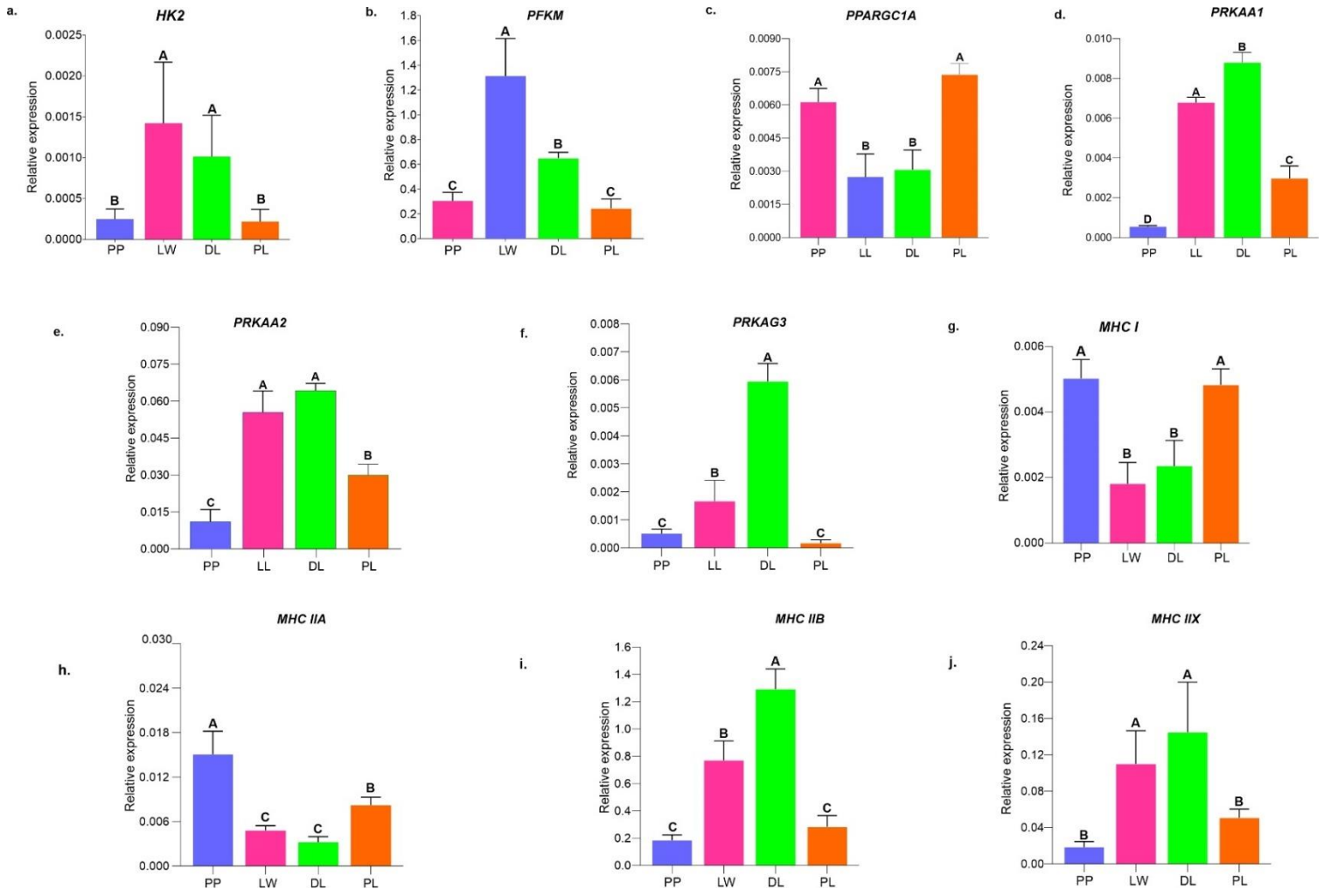

Figure 2. Analysis of relative gene expression of transcripts expressed at time 0, in pure and crossed lines.

Glycolytic genes and energy metabolism (a, b). Metabolic regulation genes (c, d, e, f). Muscle isoforms (g, h, i, j). Averages are relative expressions, described in arbitrary units. The letters represent significant differences between the group. The error bar in the figure represents the standard deviation (SD). PP, Piau; LW, Large White; DL, Duroc-Large White and PL, Piau-Large White. Significance levels Tukey test $p<0.05$.

3.3 *Gene ontology and KEGG pathway analysis for genes in study*

The teen genes were mapped to the STRING program, and 54 significantly different GO terms were obtained (p<0.05). These terms were categorized into three groups: biological processes (33 GO terms), molecular functions (16 GO terms), and cellular components (5 GO terms) (Supplementary Table 1).

These were significantly enriched in twenty-five KEGG pathways (p<0.05), with the most influenced pathway being protein localization to lipid droplet. Well-known pathways affecting muscle fiber transition (AMPK signaling pathway), muscle development (insulin signaling pathway) lipid metabolism (adipocytokine signaling pathway, mTOR signaling pathway), and energy metabolism regulation (apelin signaling pathway, FoxO signaling pathway, glycolysis / gluconeogenesis, glucagon signaling pathway) were enriched in muscle LD swine (Figure 3).

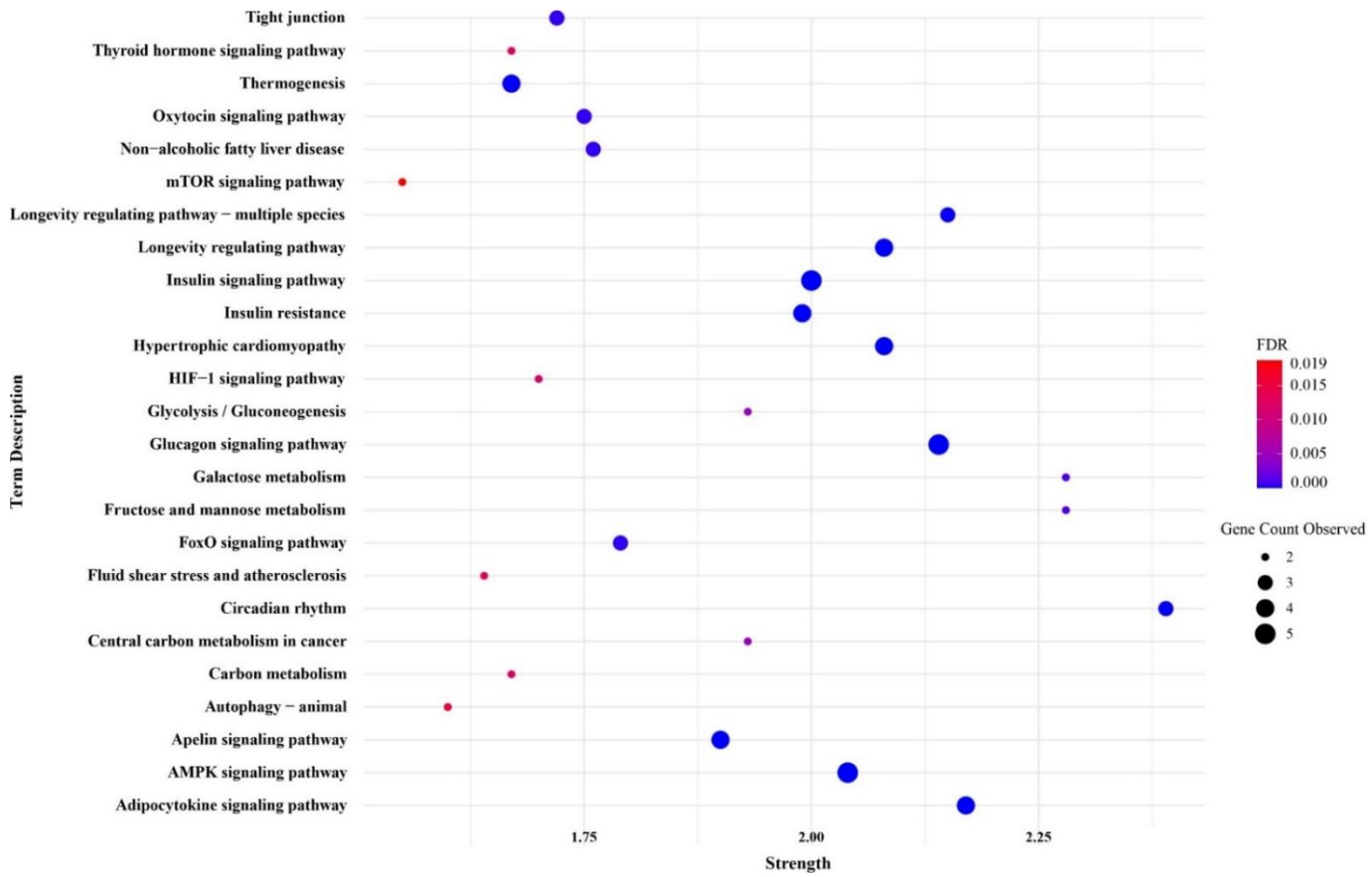

Figure 3. Enriched KEGG pathway for genes under study in swine LD muscle.

X axis, strength of genes ranging from 1.55 to 2.39 log10 units; Y axis, term description of biological processes. Gene counts observed range from 2 to 5 and are represented by the size of the bubbles. Significance level measured according to the FDR value (<0.05). Blue represents high significance (FDR < 0.005). Red indicates values approaching the significance threshold (FDR < 0.05).

3.4 ***Correlation among pH values and differentially expressed genes***

Spearman correlation was calculated to evaluate the relationship among the ten genes and between these ten genes and eleven pH values within each genetic group. The results revealed some significant correlations (ρs) when evaluated with a 5% *p-value* threshold, which was classified as: null (r=0), weak (0-0.30), moderate (0.31-0.60), strong (0.61-0.90), very strong (0.91-0.99), and perfect (r=1.0), according to Callegari, 2009. In Figure 4, the correlations are illustrated through a heatmap, which display distinct patterns of association between genes.

The Large White breed also showed a weak positive correlation (p<0.05) between *PPARGC1A* and pH0.75, *PRKAA2* and myosin isoform *MHC IIX,* between pH1 and pH2. A weak negative correlation between *MHC IIA* and pH0.75 and between pH1 and pH8. Moderate positive correlations were observed between *PFKM* with *HK2* and *MHC IIB*, between *PPARGC1A* with *PRKAA2* and pH6, *PRKAG3* and pH2, *MHC IIA* with pH5 and pH24, among myosin isoforms *MHC IIB* and *MHC IIX,* and between pH values in time zero(pH0) with pH7 and pH24. Additionally, moderate negative correlation was found between the myosin isoform *MHC I* and pH3. There was a strong positive correlation between *HK2* and pH4, *PPARGC1A* and *MHC IIA* and between *PRKAA2* with pH3, pH6 and pH24 (Figure 4).

The Duroc-Large White cross showed significant correlations (p<0.05) of the strong positive type between *MHC I* with pH6 and pH7, between myosin isoforms *MHC IIA* and *MHC IIB,* and among pH3 and pH4. However, strong negative correlation was observed between *HK2* and pH1. Moderate negative correlation was found between *HK2* and *PFKM, PPARGC1A* and pH0.75, *PRKAA1* and pH1, *PRKAA2* and pH at time zero (pH0), and between *MHC IIX* and pH2. Moderate positive was found between *PRKAA2* and pH7, *MHC I* and pH3, and between pH0.75 and pH8.

Weak positive correlations were observed between *HK2* and *MHC IIB*, *PRKAA1* and pH2 and between *PRKAG3* with pH5, pH6 and pH8. In contrast, weak negative correlation was found between pH1 and pH7 (Figure 4).

When analyzing the PL cross, we observed weak and positive correlations between *PPARGC1A* with pH24, *PRKAA2* and pH6, *PRKAG3* and myosin isoform *MHC IIX,* pH0 and pH6 and between pH0.75 and pH4. In contrast, *PRKAA1* and pH6, as well as *MHC IIB* and pH0.75, demonstrated weak but negative correlations (p<0.05).

Additionally, moderate negative correlations were observed between *HK2* with pH0 and pH6, *PFKM* and pH0 and between *PRKAA1* and pH8. Along with a moderate positive correlation between the *PFKM* and *MHC IIB, PRKAG3* and pH0.75, and between pH7 and pH in time 24 hours (pH24). There was also a strong positive correlation among myosin isoforms *MHC IIA* and pH24, and a strong but negative correlation between *HK2* and pH24, as detailed in Figure 4.

For the local breed, PP, significant correlation values were identified (p<0.05) mostly classified as weak positive, between: *HK2* with pH4 and pH7, *MHC IIX* with *MHC IIB* and pH0.75, as well as between pH7 and pH24. As still classified as week, but negative: *HK2* and pH0, *PRKAG3* with pH0 and pH4 and between *MHC IIB* with pH7 and pH24.

In addition, moderate positive correlations were observed between *PRKAA1* and pH8 and among pH0 and pH7. A strong positive correlation was obtained between pH0 and pH1, pH2 with pH6 and pH8. Furthermore, a perfect positive correlation between pH at time 5 (pH5) and pH at time 7 (pH7) was also observed, as detailed in Figure 4.

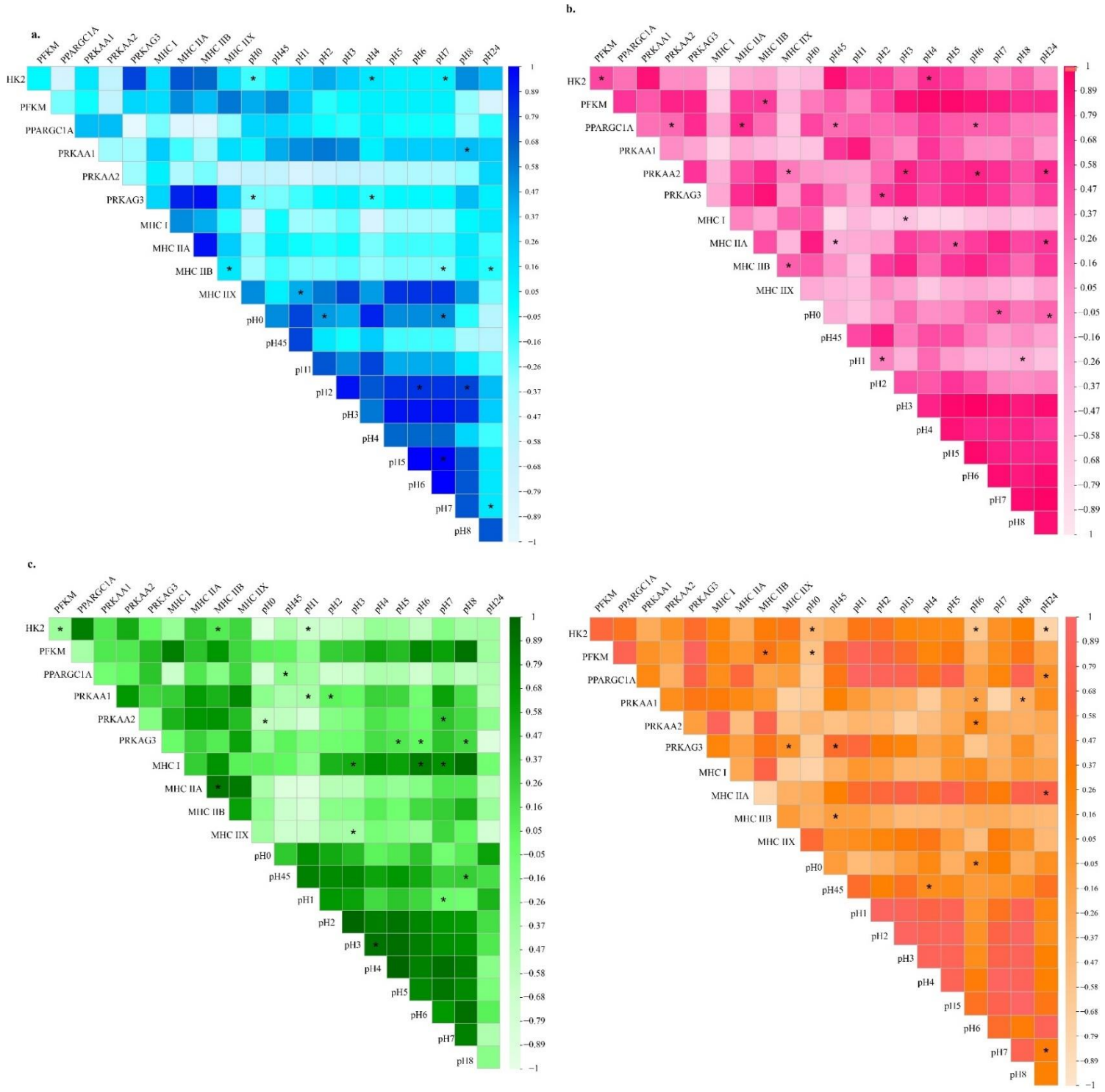

Figure 4. Heatmap with the correlation between muscle pH at time zero and genes, in the different swine genetic groups.

The letter a. represents the PP cross, b. the LW cross, c. the DL cross, and d. the PL cross. The heatmap was built in the R software, using the lighter color scale representing negative correlation, while darker colors represent positive correlation between genes or between pH0 and genes. Asterisks within each square indicate the existence of a significant correlation, p-value<0.05

4. DISCUSSION

The quality of pork is influenced by multiple factors such as pH value, intramuscular fat (IMF), tenderness, color, and water-holding capacity (WHC) (Sampath *et al.,* 2021). These characteristics vary significantly between breeds and during the *post-mortem* muscle glycolytic process (Shen *et al.,* 2014).

During the process of glycogen degradation into lactate *post-mortem* releases hydrogen ions, which significantly impact muscle pH, particularly at 0.75- and 24-hours post-slaughter. The rate and extent of pH decline are crucial for meat quality because they affect the inactivation of glycolytic enzymes (Warner *et al.,* 2022). In this study, the Piau breed consistently exhibited higher pH values at all measurement points, whereas the Large White breed showed the lowest pH values (Figure 1 and Table 4).

The elevated pH in Piau can be attributed to a lower contribution from glycolysis and lactic acid production for energy (ATP), coupled with reduced muscle glycogen stores. This is supported by the negative linear relationship between glycogen stores and pH observed in previous research (Henckel *et al.,* 2002). Additional studies have shown that oxidative muscles, which contain a higher proportion of slow-twitch fibers, typically have lower glycogen stores compared to glycolytic muscles. The reduced glycogen reserves lead to a diminished rate of post-mortem glycolysis, resulting in a higher final pH in pork (England *et al.,* 2016).

In this study, the Piau breed exhibited higher pH values across all eleven time points measured. This can be attributed to the breed's higher oxidative capacity in skeletal muscle. Specifically, these pigs have lower muscle glycogen content and a higher proportion of oxidative muscle fibers (Gilbert *et al.,* 2007; Yu *et al.,* 2013), which results in higher final pH values, as shown in Table 4 and Figure 1.

Another factor contributing to the higher pH values is the microbial flora of the pigs (Han *et al.,* 2020). Although this study does not focus on that aspect, it is known that local breeds have a greater ability to utilize dietary fibers due to their adaptation, which improves digestion and nutrient absorption by intestinal microorganisms. This adaptation leads to changes in muscle glycogen storage and contributes to higher final pH values.

In contrast, the Piau-Large White crossbreed shows greater similarity to the commercial Large White breed, with lower muscle pH values and a lower pH24 compared to

the local Piau breed. This highlights the influence of the Large White breed in the cross, as it has a higher glycogen content, which can lead to a significant decrease in muscle pH (Lefaucheur *et al.,* 2011; Smith *et al.,* 2011) (Figure 1 and Table 4).

One of the most important processes during muscle to meat transformation is the decline in pH. The rate of pH decline is crucial in determining meat quality parameters because it is closely related to the pH values and the isoelectric point of muscle proteins. This relationship affects exudate loss due to reduced water molecule attraction and decreased protein solubility (Huff-Lonergan and Lonergan, 2005; Kang *et al.,* 2021).

The results indicate that the DL cross exhibited a higher rate of pH decline compared to the local Piau breed (PP) and its cross (PL), which showed a better pH decline (Figure 1). This can be attributed to the higher glycolytic potential of the commercial DL breed. Additionally, other factors may influence this outcome, such as residual glycogen levels, glucose, Glu-6-P, and lactate levels (Xie *et al.,* 2023). Furthermore, the fat content in the tissue also plays a crucial role in pH decline. The PP breed, with its higher intramuscular fat (IMF) content, faces challenges in pH decline due to difficulties in temperature reduction, which in turn affects pH values (England *et al.,* 2016).

The hexokinase 2 (*HK2*) and muscle phosphofructokinase (*PFKM*) are pivotal enzymes in the glycolytic pathway, playing critical roles in the regulation of glucose metabolism. *HK2*, as the initiating enzyme of this pathway, catalyzes the first step in glucose metabolism by phosphorylating glucose to glucose-6-phosphate. This step is crucial because it essentially traps glucose within the cell, allowing it to be further metabolized. Notably, among the three isoforms identified in swine *HK1*, *HK2*, and *HK3*. *HK2* has emerged as the predominantly differentially expressed gene, particularly in certain muscle types (Rabbani and Thornalley, 2024). Its association with the outer mitochondrial membrane via the voltage-dependent anion channel (VDAC) not only enhances *HK2* activity but also accelerates the overall glycolytic rate (Ni *et al.,* 2024).

PFKM, on the other hand, serves as a rate-limiting enzyme in glycolysis. It catalyzes the conversion of fructose-6-phosphate from glucose-6-phosphate, a step tightly regulated by multiple allosteric effectors, including ATP, which inhibits PFKM activity (England *et al.,* 2014). The interplay between *HK2* and *PFKM* is fundamental, as both enzymes govern key control points in glycolysis, thereby influencing glucose flux through the pathway.

Our findings suggest that the higher expression of *HK2* in the LW group, followed by the DL group, correlates with the more pronounced pH decline observed in the Duroc-Large White cross (Figure 2). This observation can be attributed to phosphorylation by AMP-activated protein kinase (AMPK), which not only increases glycolytic flux by enhancing the AMP/ATP ratio but also influences muscle fiber characteristics through its interaction with myofibrillar proteins, thereby affecting catalytic activity (Ren *et al.,* 2022). Nevertheless, the lower final pH observed in the DL group despite similar *HK2* expression levels highlights the complexity of glycolytic regulation, where enzymatic expression alone is insufficient. Factors such as substrate availability and fiber type composition also play crucial roles (Figure 1 and Figure 2).

In comparison, local animals and the PL cross exhibited consistently lower expression levels for *HK2* and *PFKM*. This observation can be explained by muscle fiber composition, where a higher proportion of oxidative fibers, which rely more on lipid metabolism than glycolysis for energy production, may lead to reduced expression of glycolytic genes. This inverse relationship between oxidative fiber type and *HK2/PFKM* expression aligns with Tan *et al.* (2023), who reported that animals with higher expression of *MHC I* and *MHC IIA*, indicative of oxidative fiber dominance, had lower levels of these glycolytic enzymes.

Still evaluating the genes, the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1α*), it exhibited greater relative expression in the *Longissimus dorsi* of the local breed (PP) and its crossbreed (PL) can be attributed to the genetic predisposition of these breeds concerning the specific properties of the gene. The transcriptional coactivator *PGC-1α* (also known as *PPARGC1A*) plays a significant role in regulating energy levels and fatty acid metabolism. Furthermore, *PGC-1α* induces mitochondrial biogenesis and the transformation of fast glycolytic fibers into slow oxidative fibers (Wu *et al.,* 1999; Lin *et al.,* 2002), processes that require a more oxidative metabolism.

Noted in local breeds, which exhibit higher intramuscular fat content and greater expression of oxidative muscle fibers, such as type I and IIA fibers, thus contributing to the metabolism required to meet the properties of the *PPARGC1A* gene, especially during hypoxia, where lipid oxidation becomes predominant due to the absence of glucose to maintain energy homeostasis (Uldry *et al.,* 2006; Zhang *et al.,* 2022).

Evaluating the heterotrimeric complex AMP-activated protein kinase (*AMPK*), characterized by a catalytic α and two non-catalytic subunits, β and γ, which plays a crucial

role in regulating cellular energy balance in animal energy metabolism (Xue and Kahn, 2006), as well as, in some cases, muscle fiber typing (Rockl *et al.,* 2007). The α-*AMPK* subunits, composed α1 and α2, encoded by the *PRKAA1* and *PRKAA2* genes, respectively, showed higher expression. In the LD muscle of Duroc-Large White and Large White pigs, both groups with lower IMF content compared to the local PP breed and its PL crossbreed. This result stems from increased lipid oxidation and glucose uptake, leading to a decrease in intramuscular fat content in pigs (Carling, 2004). Thus, animals with lower IMF exhibit lower *AMPK* activity in the porcine skeletal muscle *(*Yao *et al.,* 2019).

Among the catalytic subunits, the α2-*AMPK* stands out due to its greater involvement in regulating glycolysis and lipid metabolism, as well as protein synthesis in peripheral tissues and animal body weight, making it an interesting candidate for fat deposition and muscle development in pigs (Lin *et al.,* 2010). It also plays an important role in skeletal muscle by promoting slow-twitch fibers, which in this case, would provide commercial animals with better meat quality (Viollet *et al.,* 2003; Graham, 2014).

Similarly, the non-catalytic subunit represented by γ3-*AMPK*, encoded by the *PRKAG3* gene, expressed exclusively in skeletal muscle, also showed higher relative expression in commercial breeds, especially in the Duroc-Large White group. This can be explained by the greater response of the AMPK complex to AMP, which would be consistent with the higher amount of muscle glycogen present in these breeds. This result is also consistent with previous reports regarding this subunit being highly expressed in fast-twitch oxidative fibers (Mahlapuu *et al.,* 2004), often being undetectable in slow-twitch oxidative fibers in mammals (Yu *et al.,* 2004). Additionally, *PRKAG3* is related to several quantitative trait loci (QTL) for muscle pH, providing a higher rate of pH decline in comparison to breeds with lower gene expression levels (Ciobanu *et al.,* 2001; Ovilo *et al.,* 2002), as observed in this work.

Certainly, lactate production is not the only metabolic process affecting the post-mortem decline in pH. According to Matarneh, Scheffler and Gerrard (2023), the complex metabolic properties of skeletal muscle tissue play a critical role in this process. The composition of muscle fiber types varies considerably depending on the muscle's location and function (Realini *et al.,* 2013). Muscle fibers are classified as slow-twitch oxidative or fast-twitch glycolytic (Brooke and Kaiser, 1970). In mammalian skeletal muscle, four main myosin isoforms have been described (I, IIA, IIB, and IIX), with all being expressed in pigs after birth (Fazarinc *et al.,* 2020).

This variation in fiber type contributes to differences in muscle pH values and, consequently, in pork quality. A higher expression of oxidative myosin isoforms (*MHC I* and *MHC IIA*) generally results in better quality pork due to more favorable pH values and a more gradual pH decline (Ryu and Kim, 2006; Moreno *et al.,* 2020; Zequan *et al.,* 2021). Conversely, more glycolytic myosin isoforms (IIB and IIX) exhibit less favorable properties (Xu *et al.,* 2022).

The present study observed significant differences in the relative expression of glycolytic (*IIB* and *IIX*) versus oxidative fibers (I and IIA). The local breed and its crossbreeding (PL) exhibited a higher level of expression of oxidative muscle fibers (*MHC I a*nd *MHC IIA*). Notably, there was a difference between the PP and PL breeds in the expression of the *MHC IIA* isoform, with the Piau-Large White cross showing variation from the local breed. Unlike commercial breeds, local pig breeds have a higher proportion of oxidative fibers (type I and IIA), partly due to their slower muscle acidification (Andrés *et al.,* 2001; Albuquerque *et al.,* 2021). This suggests a less glycolytic metabolism in these animals compared to commercial breeds (LW and DL), which results in higher final pH values and pH0.75, as previously discussed.

These findings align with those of Wu *et al.* (2024), who, although not using the Piau breed specifically, studied a Chinese local breed reflecting a greater expression of oxidative isoforms relative to glycolytic ones (*MHC IIB* and *MHC IIX*). This is associated with the breed's slower growth rate (Table 2), higher fat content, and more oxidative metabolism, as these fibers are associated with higher lipid content (Listrat *et al.,* 2016).

In contrast, the abundance of glycolytic isoforms (*MHC IIB* and *MHC IIX*) is typically observed in pigs selected for leanness and high performance (Listrat *et al.,* 2016). However, this abundance can negatively impact meat quality, as it induces shifts in muscle metabolism towards a more glycolytic profile at the expense of oxidative fibers. This results in lower pH values, as observed and discussed in this study (Table 4), and affects other parameters such as drip loss and color (Choi *et al.,* 2017; Zequan *et al.,* 2022).

Among the significantly enriched KEGG pathways (Figure 3), all identified pathways are important in this study. *AMPK* signaling pathways, which play a crucial role in metabolism control and downstream target regulation (Hsu *et al.,* 2022), energy metabolism pathways (Rui, 2014) such as Glycolysis/Gluconeogenesis, the Thermogenesis pathway, as

well as the mTOR pathway, which is important in protein biosynthesis, growth, and proliferation processes in mammals (Cantó and Auwerx, 2010; Yang *et al.,* 2023).

Additionally, the mTOR pathway it promotes the transcription of oxidative to glycolytic myofibers, thereby increasing the expression of glycolytic enzymes like *HK2* and *PFKM* (Baumert *et al.*, 2024; Li *et al.,* 2024). Furthermore, considering the evidence of genes in the hypoxia pathway, the enzymes involved in glucose uptake and glycolysis can be related (Jiang *et al.,* 2024), while the observation of glucagon and insulin pathways highlights their importance in maintaining animal glucose homeostasis by releasing counterregulatory hormones (Gradel *et al.,* 2020).

5. CONCLUSION

Among the analyzed genes, the expression of myosin isoforms, particularly *MHC I* and *MHC IIB*, stands out due to the notable differences observed between local and commercial breeds. Additionally, the subunits of the AMPK complex, may play a role in enhancing in pork quality.

The crossbred animals exhibited predominantly additive traits, retaining characteristics from local breeds, such as higher pH and slower pH decline. This suggests a promising opportunity to integrate the meat quality of local breeds with the productivity of commercial breeds. It is clear that pork quality parameters are polygenic traits influenced by metabolic and enzymatic processes. These include interactions between muscle fiber types, pH values, especially at 0.75 (45 minutes) and 24 hours *post-mortem*, and the rate of pH decline. Such factors can lead to excessive proteolysis and consequently deterioration in meat quality.

Incorporating local breeds into crossbreeding programs, alongside research to pinpoint specific loci associated with meat quality, represents a valuable strategy. This approach could effectively combine the advantageous traits of local breeds with the productivity benefits of commercial breeds, ultimately improving pork characteristics.

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7. APPENDIX

Supplementary Table 1. GO analyses

Biological Process

Term ID	Term description	Observed gene count	Background gene count	Strength	False discovery rate	Matching proteins in your network (IDs)	Matching proteins in your network (labels)*
GO:0014823	Response to activity	4	22	2.63	2.47E-06	9823.ENSSSCP00000017163, 9823.ENSSSCP00000032437, 9823.ENSSSCP00000049395, 9823.ENSSSCP00000055569	PRKAG3, PPARGC1A, MYH1, PRKAA2
GO:0006006	Glucose metabolic process	4	84	2.05	0.00019	9823.ENSSSCP00000008819, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000031307, 9823.ENSSSCP00000032437	HK2, PRKAA1, PFKM, PPARGC1A
GO:0014850	Response to muscle activity	3	14	2.7	0.00019	9823.ENSSSCP00000017163, 9823.ENSSSCP00000032437, 9823.ENSSSCP00000055569	PRKAG3, PPARGC1A, PRKAA2
GO:0005975	Carbohydrate metabolic process	5	388	1.48	0.00046	9823.ENSSSCP00000008819, 9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000031307, 9823.ENSSSCP00000032437	HK2, PRKAG3, PRKAA1, PFKM, PPARGC1A
GO:0032787	Monocarboxylic acid metabolic process	5	398	1.47	0.00046	9823.ENSSSCP00000008819, 9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000031307, 9823.ENSSSCP00000055569	HK2, PRKAG3, PRKAA1, PFKM, PRKAA2
GO:0006096	Glycolytic process	3	38	2.27	0.00078	9823.ENSSSCP00000008819, 9823.ENSSSCP00000017163, 9823.ENSSSCP00000031307	HK2, PRKAG3, PFKM
GO:0042149	Cellular response to glucose starvation	3	42	2.23	0.00083	9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	PRKAG3, PRKAA1, PRKAA2
GO:0001678	Cellular glucose homeostasis	3	51	2.14	0.0011	9823.ENSSSCP00000008819, 9823.ENSSSCP00000024198,	HK2, PRKAA1, PRKAA2

						9823.ENSSSCP00000055569	
GO:1990044	Protein localization to lipid droplet	2	4	3.07	0.0014	9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	PRKAA1, PRKAA2
GO:0062028	Regulation of stress granule assembly	2	5	2.98	0.0019	9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	PRKAA1, PRKAA2
GO:1904428	Negative regulation of tubulin deacetylation	2	5	2.98	0.0019	9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	PRKAA1, PRKAA2
GO:0006091	Generation of precursor metabolites and energy	4	293	1.51	0.002	9823.ENSSSCP00000008819, 9823.ENSSSCP00000017163, 9823.ENSSSCP00000031307, 9823.ENSSSCP00000032437	HK2, PRKAG3, PFKM, PPARGC1A
GO:0044281	Small molecule metabolic process	6	1328	1.03	0.002	9823.ENSSSCP00000008819, 9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000031307, 9823.ENSSSCP00000032437, 9823.ENSSSCP00000055569	HK2, PRKAG3, PRKAA1, PFKM, PPARGC1A, PRKAA2
GO:1905691	Lipid droplet disassembly	2	6	2.9	0.002	9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	PRKAA1, PRKAA2
GO:0031667	Response to nutrient levels	4	325	1.46	0.0026	9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000032437, 9823.ENSSSCP00000055569	PRKAG3, PRKAA1, PPARGC1A, PRKAA2
GO:0042752	Regulation of circadian rhythm	3	93	1.88	0.0027	9823.ENSSSCP00000024198, 9823.ENSSSCP00000032437, 9823.ENSSSCP00000055569	PRKAA1, PPARGC1A, PRKAA2
GO:0006002	Fructose 6-phosphate metabolic process	2	9	2.72	0.0029	9823.ENSSSCP00000008819, 9823.ENSSSCP00000031307	HK2, PFKM
GO:0016310	Phosphorylation	5	863	1.14	0.0039	9823.ENSSSCP00000008819, 9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000031307, 9823.ENSSSCP00000055569	HK2, PRKAG3, PRKAA1, PFKM, PRKAA2
GO:0071380	Cellular response to prostaglandin E stimulus	2	11	2.63	0.0039	9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	PRKAA1, PRKAA2
GO:0044262	Cellular carbohydrate metabolic process	3	138	1.71	0.0059	9823.ENSSSCP00000008819, 9823.ENSSSCP00000017163, 9823.ENSSSCP00000031307	HK2, PRKAG3, PFKM

GO:0006936	Muscle contraction	3	144	1.69	0.006	9823.ENSSSCP00000049395, 9823.ENSSSCP00000061002, 9823.ENSSSCP00000070632	MYH1, MYH4, MYH7
GO:0034599	Cellular response to oxidative stress	3	143	1.7	0.006	9823.ENSSSCP00000024198, 9823.ENSSSCP00000032437, 9823.ENSSSCP00000055569	PRKAA1, PPARGC1A, PRKAA2
GO:1901863	Positive regulation of muscle tissue development	2	19	2.4	0.0073	9823.ENSSSCP00000024198, 9823.ENSSSCP00000032437	PRKAA1, PPARGC1A
GO:0048511	Rhythmic process	3	167	1.63	0.0088	9823.ENSSSCP00000024198, 9823.ENSSSCP00000032437, 9823.ENSSSCP00000055569	PRKAA1, PPARGC1A, PRKAA2
GO:2000758	Positive regulation of peptidyl-lysine acetylation	2	31	2.18	0.0164	9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	PRKAA1, PRKAA2
GO:0071333	Cellular response to glucose stimulus	2	32	2.17	0.0169	9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	PRKAA1, PRKAA2
GO:0032007	Negative regulation of TOR signaling	2	39	2.08	0.021	9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	PRKAA1, PRKAA2
GO:0033554	Cellular response to stress	5	1404	0.93	0.021	9823.ENSSSCP00000008819, 9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000032437, 9823.ENSSSCP00000055569	HK2, PRKAG3, PRKAA1, PPARGC1A, PRKAA2
GO:0006631	Fatty acid metabolic process	3	258	1.44	0.0229	9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	PRKAG3, PRKAA1, PRKAA2
GO:0070252	Actin-mediated cell contraction	2	42	2.05	0.0229	9823.ENSSSCP00000049395, 9823.ENSSSCP00000070632	MYH1, MYH7
GO:0006950	Response to stress	6	2589	0.74	0.0288	9823.ENSSSCP00000008819, 9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000032437, 9823.ENSSSCP00000049395, 9823.ENSSSCP00000055569	HK2, PRKAG3, PRKAA1, PPARGC1A, MYH1, PRKAA2
GO:0044283	Small molecule biosynthetic process	3	347	1.31	0.0434	9823.ENSSSCP00000017163, 9823.ENSSSCP00000032437, 9823.ENSSSCP00000055569	PRKAG3, PPARGC1A, PRKAA2
GO:0071277	Cellular response to calcium ion	2	66	1.86	0.0441	9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	PRKAA1, PRKAA2

33 terms

Molecular Function

Term ID	Term description	Observed gene count	Background gene count	Strength	False discovery rate	Matching proteins in your network (IDs)	Matching proteins in your network (labels)*
GO:0004679	AMP-activated protein kinase activity	3	7	3.01	9.82E-06	9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	PRKAG3, PRKAA1, PRKAA2
GO:0005524	ATP binding	8	1410	1.13	9.82E-06	9823.ENSSSCP00000008819, 9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000031307, 9823.ENSSSCP00000049395, 9823.ENSSSCP00000055569, 9823.ENSSSCP00000061002, 9823.ENSSSCP00000070632	HK2, PRKAG3, PRKAA1, PFKM, MYH1, PRKAA2, MYH4, MYH7
GO:0000146	Microfilament motor activity	3	34	2.32	8.77E-05	9823.ENSSSCP00000049395, 9823.ENSSSCP00000061002, 9823.ENSSSCP00000070632	MYH1, MYH4, MYH7
GO:0047322	[hydroxymethylglutaryl-CoA reductase (NADPH)] kinase activity	2	3	3.2	0.00032	9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	PRKAA1, PRKAA2
GO:0050405	[acetyl-CoA carboxylase] kinase activity	2	3	3.2	0.00032	9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	PRKAA1, PRKAA2
GO:0016773	Phosphotransferase activity, alcohol group as acceptor	5	681	1.24	0.00069	9823.ENSSSCP00000008819, 9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000031307, 9823.ENSSSCP00000055569	HK2, PRKAG3, PRKAA1, PFKM, PRKAA2
GO:0016301	Kinase activity	5	757	1.19	0.0011	9823.ENSSSCP00000008819, 9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000031307, 9823.ENSSSCP00000055569	HK2, PRKAG3, PRKAA1, PFKM, PRKAA2
GO:1901363	Heterocyclic compound binding	9	5735	0.57	0.0012	9823.ENSSSCP00000008819, 9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000031307,	HK2, PRKAG3, PRKAA1, PFKM, PPARGC1A, MYH1, PRKAA2, MYH4, MYH7

						9823.ENSSSCP00000032437, 9823.ENSSSCP00000049395, 9823.ENSSSCP00000055569, 9823.ENSSSCP00000061002, 9823.ENSSSCP00000070632	
GO:0035174	Histone serine kinase activity	2	9	2.72	0.0013	9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	PRKAA1, PRKAA2
GO:0097159	Organic cyclic compound binding	9	5810	0.56	0.0013	9823.ENSSSCP00000008819, 9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000031307, 9823.ENSSSCP00000032437, 9823.ENSSSCP00000049395, 9823.ENSSSCP00000055569, 9823.ENSSSCP00000061002, 9823.ENSSSCP00000070632	HK2, PRKAG3, PRKAA1, PFKM, PPARGC1A, MYH1, PRKAA2, MYH4, MYH7
GO:0016208	AMP binding	2	11	2.63	0.0017	9823.ENSSSCP00000017163, 9823.ENSSSCP00000031307	PRKAG3, PFKM
GO:0005516	Calmodulin binding	3	143	1.7	0.0031	9823.ENSSSCP00000049395, 9823.ENSSSCP00000061002, 9823.ENSSSCP00000070632	MYH1, MYH4, MYH7
GO:0019200	Carbohydrate kinase activity	2	20	2.37	0.0041	9823.ENSSSCP00000008819, 9823.ENSSSCP00000031307	HK2, PFKM
GO:0051015	Actin filament binding	3	206	1.54	0.0081	9823.ENSSSCP00000049395, 9823.ENSSSCP00000061002, 9823.ENSSSCP00000070632	MYH1, MYH4, MYH7
GO:0048029	Monosaccharide binding	2	62	1.88	0.0321	9823.ENSSSCP00000008819, 9823.ENSSSCP00000031307	HK2, PFKM
GO:0003725	Double-stranded RNA binding	2	66	1.86	0.0351	9823.ENSSSCP00000049395, 9823.ENSSSCP00000061002	MYH1, MYH4
16 terms							

Cellular Components

Term ID	Term description	Observed gene count	Background gene count	Strength	False discovery rate	Matching proteins in your network (IDs)	Matching proteins in your network (labels)*
GO:0031588	Nucleotide-activated protein kinase	3	9	2.9	1.81E-05	9823.ENSSSCP00000017163,	PRKAG3, PRKAA1, PRKAA2

	complex					9823.ENSSSCP00000024198, 9823.ENSSSCP00000055569	
GO:0016460	Myosin II complex	3	17	2.62	3.97E-05	9823.ENSSSCP00000049395, 9823.ENSSSCP00000061002, 9823.ENSSSCP00000070632	MYH1, MYH4, MYH7
GO:0032982	Myosin filament	3	16	2.65	3.97E-05	9823.ENSSSCP00000049395, 9823.ENSSSCP00000061002, 9823.ENSSSCP00000070632	MYH1, MYH4, MYH7
GO:0061695	Transferase complex, transferring phosphorus-containing groups	4	253	1.57	0.00079	9823.ENSSSCP00000017163, 9823.ENSSSCP00000024198, 9823.ENSSSCP00000031307, 9823.ENSSSCP00000055569	PRKAG3, PRKAA1, PFKM, PRKAA2
GO:0030016	Myofibril	3	173	1.61	0.0101	9823.ENSSSCP00000049395, 9823.ENSSSCP00000061002, 9823.ENSSSCP00000070632	MYH1, MYH4, MYH7
5 terms							

*MYH1: MHC IIX; MYH4: MHC IIB; MYH7: MHC I